Int. J. Devl Neuroscience 31 (2013) 96–104

Contents lists available at SciVerse ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

# Gestational doxorubicin alters fetal thyroid–brain axis

R.G. Ahmed [a,*], S. Incerpi [b]

[a] Division of Anatomy and Embryology, Zoology Department, Faculty of Science, Beni-Suef University, Egypt
[b] Department of Biology, University Roma Tre, Viale Marconi 446, 00146 Roma, Italy

ARTICLE INFO

*Article history:*
Received 11 July 2012
Received in revised form
15 November 2012
Accepted 16 November 2012

*Keywords:*
Doxorubicin
Thyroid
Cerebrum
Cerebellum
Nucleotides
ATPases
Adenylate system
Fetus
Pregnant rats

ABSTRACT

Administration of chemotherapy during pregnancy may represent a big risk factor for the developing brain, therefore we studied whether the transplacental transport of doxorubicin (DOX) may affect the development of neuroendocrine system. DOX (25 mg/kg; 3 times interaperitoneally/week) was given to pregnant rats during whole gestation period. The disturbances in neuroendocrine functions were investigated at gestation day (GD) 15 and 20 by following the maternal and fetal thyroid hormone levels, fetal nucleotides (ATP, ADP, AMP) levels and adenosine triphosphatase ($Na^+$, $K^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase) activities in two brain regions, cerebrum and cerebellum. In control group, the levels of maternal and fetal serum thyroxine (T4), triiodothyronine (T3), thyrotropin (TSH), and fetal serum growth hormone (GH) increased from days 15 to 20, whereas in the DOX group, a decrease in maternal and fetal T4, T3 and increase in TSH levels (hypothyroid status) were observed. Also, the levels of fetal GH decreased continuously from GD 15 to 20 with respect to control group. In cerebrum and cerebellum, the levels of fetal nucleotides and the activities of fetal ATPases in control group followed a synchronized course of development. The fetal hypothyroidism due to maternal administration of DOX decreased the levels of nucleotides, ATPases activities, and total adenylate, instead, the adenylate energy charge showed a trend to an increase in both brain regions at all ages tested. These alterations were dose- and age-dependent and this, in turn, may impair the nerve transmission. Finally, DOX may act as neuroendocrine disruptor causing hypothyroidism and fetal brain energetic dysfunction.

© 2012 ISDN. Published by Elsevier Ltd. All rights reserved.

## 1. Introduction

The anthracycline antibiotic, doxorubicin (DOX), is an anticancer drug that is widely used in the treatment of leukemias, lymphomas, lung, breast, ovary and uterine cancers (Kumar et al., 2011). However, there are several side effects related to DOX chemotherapy. Injection of DOX in rats, at midgestation and at term, caused thyroid agenesis, hypoplasia and disorganization (Menegola et al., 2001). Moreover, human case study reports and preclinical data in animal models indicate that DOX treatment cause mitochondrial damage and decrease the adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio (Kawasaki et al., 1996). This is due to inhibition of the calcium adenosine triphosphatase ($Ca^{2+}$-ATPase) pump (Arai et al., 2000). The use of chemotherapeutics during the fetal phase of pregnancy might affect all tissues including the nervous system, in which organogenesis proceeds well into the early postnatal period (Van Calsteren et al., 2009). As the blood brain barrier (BBB) is not yet functional in the fetal and perinatal central nervous system (CNS), any drug that cross the placenta may also reach the fetal CNS (Bigotte and Olsson, 1984; Van Calsteren et al., 2009). In general, the use of anthracyclins during pregnancy may cause a unique set of nervous system damage and neurological side effects in children or adults (Van Calsteren et al., 2009). But again, the impacts of chemotherapeutic drugs on the developing fetus, particularly brain, need to be characterized in detail.

Thyroid hormones (THs) play a pivotal role in vertebrate brain development, from early embryogenesis to subsequent development (Zoeller, 2004, 2006, 2007, 2008; Bruno et al., 2005; Gilbert and Sui, 2006; Carageorgiou et al., 2007; Ahmed et al., 2008; Elbakry et al., 2010; Horn and Heuer, 2010; Ahmed, 2012a), and are involved in the maintenance of the ionic gradient essential for neuronal excitability (Ahmed et al., 2008; Ahmed, 2012a). For all these reasons, we aimed to study the relationship between the transplacental transport of DOX to fetal thyroid–brain axis and the possible detrimental effect on the development of this axis, which is still matter of debate. The present study was carried out on pregnant albino rats with the following objectives: (1) to assess the functioning of the maternal–fetal thyroid axis; (2) to examine the effects of the administration of maternal DOX on the development of fetal neuroendocrine system; and (3) to verify the effect of maternal DOX

* Corresponding author.
*E-mail addresses:* ragabb900@yahoo.com, ahmedragab08@gmail.com (R.G. Ahmed).

0736-5748/$36.00 © 2012 ISDN. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijdevneu.2012.11.005

and THs on the levels of fetal high energy phosphate derivatives (ATP, ADP, and AMP), the activities of fetal adenosine triphosphatases ($Na^+$, $K^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase), the total adenylate (TA) system and adenylate energy charge (AEC), biomarkers of health in non-lethal stress situations (De Luca-Abbott et al., 2000) in cerebrum and cerebellum. We used rats because their placenta is very similar to the human placenta (Barber, 1981; Cardonick and Iacobucci, 2004), and also because their brain is highly sensitive to any stress (TH disturbance) during early development (Koibuchi and Chin, 2000; Zoeller and Rovet, 2004; Zoeller and Crofton, 2005; Ahmed et al., 2008, 2010, 2012; Koibuchi, 2009; Ahmed, 2012a).

## 2. Materials and methods

### 2.1. Chemicals

Doxorubicin (Adriblastina® produced by Carlo Erba, Rodano (Milano), Italy) was purchased from local pharmacy in the form of 10 mg/ampoule. Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) while adenosine triphosphate (sodium salt; ATP-$Na_2$) was purchased from Riedel De Haen AG, Germany. Thyroxine (T4), triiodothyronine (T3), thyrotropin (TSH) and growth hormone (GH) kits were obtained from Calbiotech Inc (CBI), USA. All other reagents were of the purest grades commercially available.

### 2.2. Experimental animals

Mature white albino rats (*Rattus norvegicus*, Wistar strain) were purchased from the National Institute of Ophthalmology, Giza, Egypt. This study was carried out on 12 mature virgin females/group weighing about 170–190 g and 6 mature males/group for mating only. They were kept under observation in the department animal house for 2 weeks to exclude any intercurrent infection and to acclimatize the new conditions. The animals were marked, housed in metal (stainless steel; 60 cm × 50 cm × 50 cm) separate bottom ventilated cages at normal atmospheric temperature (23 ± 2 °C) and fed on standard rodent pellet diet manufactured by the Egyptian Company for oil and soap as well as some vegetables as a source of vitamins (Ahmed et al., 2010, 2012; El-bakry et al., 2010). Tap water was used for drinking ad libitum and these animals were exposed to constant daily light/dark periods of 12 h each (lights on at 06:00 h) and 50 ± 5% relative humidity (El-bakry et al., 2010; Ahmed et al., 2012). All animal procedures are in accordance with the general guidelines of animal care and the recommendations of the Canadian Committee for Care and use of animals (Canadian Council on Animal Care, 1993). All efforts were made to minimize the number of animals used and their suffering.

Daily examination of vaginal smears of each virgin female was carried out to determine the estrous cycle. Estrous females exhibited the presence of cornified cells in vaginal smears. Mating was induced by housing proestrous females with male in separate cage at ratio of two females and one male overnight for one or two consecutive days. In the next morning, the presence of sperm in vaginal smears determined the first day of gestation. Then, the pregnant females were transferred into separate cages from males to start the experiment.

### 2.3. Experimental strategy

The dose of doxorubicin (DOX) was 25 mg/kg body weight. This dose was previously reported to include an increase in the frequency of cell damage in mammalian systems (Antunes and Takhashi, 1999; Prahalathan et al., 2006; Hozayen and Abou Seif, 2011; Hozayen, 2012). The animals were treated with DOX by intraperitoneal injection. The chosen dose of DOX was adjusted to 0.2 ml/25 g b.wt. in sterile distilled water prior to use and was given three times per week during whole gestation period. The control group was given sterile distilled water. Dams and their fetuses were decapitated under mild diethyl ether anesthesia and sampled at gestation days (GDs) 15 and 20.

The mother blood samples (6 per group) were taken from jugular vein and fetal blood samples (6 per group) were collected directly from the umbilical cord during the gestational period at days 15 and 20 (before birth or delivery). The clotted blood samples were centrifuged at speed 3000 r.p.m. (1006.2 × *g*) and at temperature 15–24 °C for 30 min. The clear, non-hemolysed supernatant sera were quickly removed, divided into three portions for each individual animal, and kept at −30 °C until use for different hormonal assays (radioimmunoassay). On the other hand, cerebrum and cerebellum of rat fetuses were quickly removed, separated and homogenized by using a Teflon homogenizer (Glas-Col, Terre Haute in USA), and kept in deep freezer at −30 °C until use for different developmental and biochemical assays.

### 2.4. The radioimmunoassay evaluation of hormones levels

The maternal and fetal serum T4, T3 and TSH, as well as fetal serum GH were estimated quantitatively in Diabetic Endocrine Metabolic Pediatric Unit, Center for Social and Preventive Medicine, New Children Hospital, Faculty of Medicine, Cairo University according to the method of Thakur et al. (1997), Maes et al. (1997), Mandel et al. (1993) and Reutens (1995), respectively.

### 2.5. The developmental and biochemical examinations in fetal brain

We estimated the global concentrations of the nucleotides (ATP, ADP and AMP) and the activities of various generating and metabolizing enzymes (adenosine 5′-triphosphatases; $Na^+$, $K^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase) found in cerebrum and cerebellum tissues at GD 15 and 20.

#### 2.5.1. Fetal ATP, ADP and AMP assays

The concentration of these nucleotides, based on ATP and ADP hydrolysis, was estimated according to the method of Chan et al. (1986) and Rico et al. (2003). The homogenates of cerebrum and cerebellum of rat fetus (5 μg protein) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ or $MgCl_2$ in a final volume of 200 μl. The samples were preincubated for 10 min at 37 °C. The reaction was initiated by the addition of substrate (ATP, ADP or AMP, as indicated) to a final concentration of 1 mM and stopped by adding 200 μl 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi). Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after mixing with TCA were used to correct non-enzymatic hydrolysis of substrates. Then, the absorbance of the final mixture was measured at 630 nm using SP 6-200 spectrophotometer. Specific activity is expressed as nmol of Pi released $min^{-1}$ mg of $protein^{-1}$. On the other hand, adenylate energy charge (AEC) has been suggested as a measure of the energy potential available from the total adenylate (TA) system of the cellular metabolism (Atkinson, 1968; El-Wardany et al., 2011), calculated from the following equations: TA = ATP + ADP + AMP and AEC = [0.5(ADP) + ATP]/TA.

#### 2.5.2. Fetal ATPases (adenosine 5′-triphosphatase) activities

The assay of the enzyme activities followed the procedure of Hesketh et al. (1978), Elekwa et al. (2005) and Ahmed et al. (2010), and monitored the Pi released from ATP. Enzyme activities were expressed as nmol of Pi released $min^{-1}$ mg of $protein^{-1}$.

(1) Sodium-potassium adenosine 5′-triphosphatase ($Na^+$, $K^+$-ATPase) activity
The reaction mixture contained 0.5 ml each of 0.35 M NaCl, 17.5 mM KCl, 21.0 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4), and 8.0 mM ATP-$Na_2$. The reaction was started by the addition of 0.2 ml of brain sample (cerebrum and cerebellum) homogenates and the mixture incubated at 37 °C for 1 h. The reaction was terminated by addition of 0.8 ml of ice-cold 10% (w/v) TCA and the resultant mixture stood for 20 min at 4 °C. It was then centrifuged at 4000 r.p.m. for 5 min using a bench-top centrifuge. Then, the concentration of Pi in 1 ml of the supernatant was measured (Fiske and Subbarow, 1925). For this, 1.0 ml of 2.5% ammonium molybdate was added and after 10 min, the addition of 0.1 ml of 2% ascorbic acid followed. The mixture was kept at room temperature for 20 min for color development. Then, the absorbance of the final mixture was measured at 725 nm using SP 6-200 spectrophotometer.
(2) Calcium adenosine 5′-triphosphatase ($Ca^{2+}$-ATPase) activity
The reaction mixture contained 0.5 ml each of 21.0 mM $MgCl_2$, 17.5 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.4), and 8.0 mM ATP-$Na_2$. The reaction was initiated and processed as described for $Na^+$, $K^+$-ATPase assay.
(3) Magnesium adenosine 5′-triphosphatase ($Mg^{2+}$-ATPase) activity
The reaction mixture contained 0.5 ml each of 21.0 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4), and 8.0 mM ATP-$Na_2$. The reaction was initiated and processed as described for $Na^+$, $K^+$-ATPase assay.

### 2.6. Statistical analysis

The data are analyzed using one-way analysis of variance (ANOVA) (PC-STAT, University of Georgia, 1985) followed by LSD analysis to discern the main effects and compare various groups with each other. *F*-probability for each variable expresses the general effect between groups. The data are presented as mean ± standard error (SE) and values of $P < 0.01$ and $P < 0.001$ are considered statistically highly significant and very highly significant, respectively.

## 3. Results

### 3.1. Maternal–fetal thyroid axis

The concentration of maternal and fetal serum thyroxine (T4), triiodothyronine (T3) and thyrotropin (TSH) in control group showed a stepwise increase from gestation day (GD) 15 to 20 (Tables 1 and 2). At GD 20, the mean values of fetal serum growth hormone (GH) of control group were significantly higher with

**Table 1**
Effect of DOX in thyroid functions [thyroxine (T4, ng/100 ml), triiodothyronine (T3, ng/100 ml), T4/T3 ratio and thyrotropin (TSH, ng/100 ml)] of pregnant rats during the gestational period.

| Periods | DXR (ml/25 g) | Serum T4 | Serum T3 | T4/T3 ratio | Serum TSH |
|---|---|---|---|---|---|
| GD 15 | 0 | $16.27 \pm 0.318^{b}$ | $1.17 \pm 0.811^{c}$ | 13.90 | $3.07 \pm 0.302^{c}$ |
| | 0.2 | $11.33 \pm 0.162^{d}$ | $0.59 \pm 0.169^{d}$ | 19.20 | $6.38 \pm 0.251^{b}$ |
| | | −30.37% | −49.58% | | +107.81% |
| GD 20 | 0 | $18.93 \pm 0.372^{a}$ | $2.99 \pm 0.530^{a}$ | 6.33 | $5.61 \pm 0.285^{b}$ |
| | 0.2 | $15.10 \pm 0.101^{c}$ | $1.65 \pm 0.152^{b}$ | 9.16 | $9.99 \pm 0.251^{a}$ |
| | | −20.23% | −44.81% | | +78.08% |
| ANOVA | | $P < 0.001$ | | | $P < 0.001$ |
| LSD 5% | | 0.769 | 0.270 | | 0.790 |
| LSD 1% | | 1.049 | 0.368 | | 1.078 |

Data are expressed as mean ± SE. Number of animals in each group is six. Values which share the same superscript symbols are not significantly different. ANOVA (*F*-probability) expresses the effect between groups, where $P < 0.001$ is very highly significant. Where, GD is gestational day.

**Table 2**
Effect of maternal DOX in thyroid functions [thyroxine (T4, ng/100 ml), triiodothyronine (T3, ng/100 ml), T4/T3 ratio, thyrotropin (TSH, ng/100 ml) and growth hormone (GH, ng/100 ml)] of their fetuses during the gestational period.

| Periods | DXR (ml/25 g) | Serum T4 | Serum T3 | T4/T3 ratio | Serum TSH | Serum GH |
|---|---|---|---|---|---|---|
| GD 15 | 0 | $10.11 \pm 0.241^{b}$ | $0.60 \pm 0.175^{c}$ | 16.85 | $1.79 \pm 0.106^{c}$ | $0.90 \pm 0.220^{c}$ |
| | 0.2 | $6.90 \pm 0.210^{c}$ | $0.32 \pm 0.254^{d}$ | 21.57 | $4.73 \pm 0.151^{b}$ | $0.60 \pm 0.290^{d}$ |
| | | −31.76% | −46.67% | | +164.24% | −33.33% |
| GD 20 | 0 | $13.67 \pm 0.239^{a}$ | $1.71 \pm 0.133^{a}$ | 8.00 | $4.43 \pm 0.196^{b}$ | $2.19 \pm 0.113^{a}$ |
| | 0.2 | $10.13 \pm 0.139^{b}$ | $0.80 \pm 0.351^{b}$ | 12.67 | $7.22 \pm 0.102^{a}$ | $1.15 \pm 0.760^{b}$ |
| | | −25.90% | −53.21% | | +62.98% | −47.49% |
| ANOVA | | $P < 0.001$ | | | | $P < 0.001$ |
| LSD 5% | | 0.628 | 0.201 | | 0.411 | 0.209 |
| LSD 1% | | 0.856 | 0.274 | | 0.560 | 0.285 |

Data are expressed as mean ± SE. Number of animals in each group is six. Values which share the same superscript symbols are not significantly different. ANOVA (*F*-probability) expresses the effect between groups, where $P < 0.001$ is very highly significant. Where, GD is gestation day.

respect to those at GD 15. The baseline levels of maternal serum T4 and T3 were decreased significantly (LSD; $P < 0.01$) below normal values in doxorubicin (DOX; 25 mg/kg) group whose serum TSH levels were significantly elevated (LSD; $P < 0.01$) (hypothyroid state) (Table 1). Interestingly, the percentage changes at GD 15 were −30.37%, −49.58% and +107.81% and at GD 20 were −20.23%, −44.81% and +78.08% for T4, T3 and TSH, respectively. The increase of maternal T4/T3 ratio was greater in DOX group with respect to control during the considered ages (Table 1).

Similarly, the levels of fetal serum T4, T3 and GH in maternal DOX group were decreased significantly (LSD; $P < 0.01$); their serum TSH levels were significantly higher (LSD; $P < 0.01$) as the age progressed from day 15 to 20 as compared with the respective controls (Table 2). Moreover, the percentage changes at GD 20 were −25.90%, −53.21%, −47.49% and +62.98% for T4, T3, GH and TSH, respectively. In addition, the inhibition in turnover of fetal T4 to T3 (increase T4/T3 ratio) was obvious in maternal DOX group as compared to corresponding control one at both GD 15 and 20 (Table 2). Tables 1 and 2 showed that the general effect on hormone levels evaluated by one-way ANOVA analysis between groups was found to be very highly significant ($P < 0.001$) throughout the experiment.

### 3.2. Fetal biochemical variables in cerebrum and cerebellum

#### 3.2.1. Fetal nucleotides

The data of the control rat fetuses indicated a gradual increase in concentration of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) in cerebrum and cerebellum from GD 15 to 20 (Fig. 1). Notably, their concentrations in cerebellum were higher if compared to cerebrum at all considered days. A highly significant decrease (LSD; $P < 0.01$) in the levels of nucleotides in rat fetuses whose mothers were treated with DOX, was found at GD 15 compared to control group in both brain regions. Furthermore, in maternal DOX group at GD 15, the decrease in the level of fetal ADP in cerebellum was greater than in cerebrum. Interestingly, the alterations in all fetal nucleotides at GD 20 were more significant in both examined brain regions of maternal treated group with respect to control ones. Also, in treated group, the decrease in the level of fetal ATP at GD 15 and 20 in cerebrum was greater than in cerebellum. In general, the depression effect of maternal DOX at GD 20 was more severe on the levels of fetal AMP (cerebrum; $9.31 \pm 0.310$ and cerebellum; $11.09 \pm 0.499$) if compared to the levels of fetal ATP (cerebrum; $42.07 \pm 0.530$ and cerebellum; $62.88 \pm 1.277$) or fetal ADP (cerebrum; $24.94 \pm 0.751$ and cerebellum; $23.83 \pm 1.543$) (Fig. 1). According to one-way ANOVA analysis, the general effect between groups of nucleotides was very highly significant ($P < 0.001$) in all studied regions (Table 3).

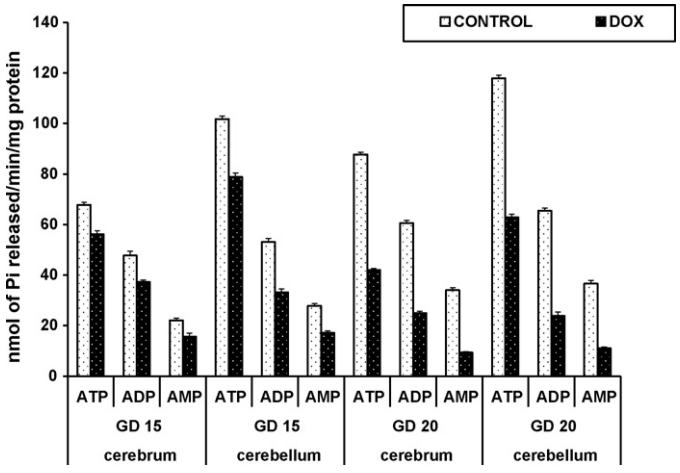


**Fig. 1.** Effect of maternal DOX on ATP, ADP and AMP hydrolysis in cerebrum and cerebellum of rat fetus at gestation days 15 and 20. Bars represent mean ± SE of six animals/group. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test are illustrated in Table 3. The change between DOX and control/parameter/period/brain region is highly significant ($P < 0.01$).

**Table 3**
LSD and ANOVA analysis for the nucleotides in cerebrum and cerebellum of rat fetuses during the gestational period.

| Brain region | Analysis | ATP | ADP | AMP |
|---|---|---|---|---|
| Cerebrum | LSD 5% | 2.973 | 3.621 | 2.904 |
| | LSD 1% | 4.056 | 4.939 | 3.961 |
| | ANOVA | | $P < 0.001$ | |
| Cerebellum | LSD 5% | 3.771 | 3.932 | 2.713 |
| | LSD 1% | 5.144 | 5.363 | 3.701 |
| | ANOVA | | $P < 0.001$ | |

ANOVA (*F*-probability) expresses the effect between groups, where $P < 0.001$ is very highly significant.

**Table 5**
LSD and ANOVA analysis for the adenosine enzymes in cerebrum and cerebellum of rat fetuses during the gestational period.

| Brain region | Analysis | $Na^+$, $K^+$-ATPase | $Ca^{2+}$-ATPase | $Mg^{2+}$-ATPase |
|---|---|---|---|---|
| Cerebrum | LSD 5% | 3.439 | 1.882 | 3.274 |
| | LSD 1% | 4.689 | 2.567 | 4.465 |
| | ANOVA | | $P < 0.001$ | |
| Cerebellum | LSD 5% | 3.812 | 3.028 | 2.902 |
| | LSD 1% | 5.199 | 4.130 | 3.959 |
| | ANOVA | | $P < 0.001$ | |

ANOVA (*F*-probability) expresses the effect between groups, where $P < 0.001$ is very highly significant.

### 3.3. Fetal total adenylate (TA) and adenylate energy charge (AEC)

The data showing changes of TA and AEC concentrations with the age and the effect of maternal DOX are shown in Table 4. The control values of fetal TA concentration were stepwisely increased in both brain regions going from GD 15 to 20. DOX administration to the dams during gestation period decreased TA to reach minimum values at GD 20 in fetal cerebrum (−58.16%) and cerebellum (−55.52%) as compared to control group. As expected, in maternal DOX group, this behavioral pattern was reversed for AEC where its levels showed a trend to an increase in treated group with respect to the age-matched controls, particularly at GD 20 where the percentage changes were +10.93% in cerebrum and +11.60% in cerebellum (Table 4).

### 3.4. Fetal adenosine triphosphatases

The adenosine triphosphatase activities of the studied brain regions in all experimental groups were allotted in Fig. 2 and Table 5. In control group, the activities of all adenosine enzymes ($Na^+$, $K^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase) were markedly increased in an age-dependent manner in fetal cerebrum and cerebellum to reach maximum values at GD 20. In control group, the activities of the $Na^+$, $K^+$-ATPase were greater in both brain regions at GD 15 and 20 if compared to the activities of $Mg^{2+}$-ATPase or $Ca^{2+}$-ATPase. In fetal cerebrum and cerebellum of maternal DOX group, the levels of all adenosine enzymes were significantly decreased (LSD; $P < 0.01$) particularly at GD 20 with respect to its own control. The depletion in their activities in cerebrum was in the following order: $Ca^{2+}$-ATPase > $Mg^{2+}$-ATPase > $Na^+$, $K^+$-ATPase and in cerebellum was in the following order: $Mg^{2+}$-ATPase > $Ca^{2+}$-ATPase > $Na^+$, $K^+$-ATPase. The analysis of one-way ANOVA showed that the general effect between groups was very highly significant ($P < 0.001$) throughout the tested brain regions (Table 5).

## 4. Discussion

The current study shows for the first time, to our knowledge, the effects of maternal doxorubicin (DOX) on the maternal and fetal thyroid functions, levels of fetal nucleotides (ATP, ADP and AMP) and activities of fetal adenosine triphosphatases ($Na^+$, $K^+$-ATPase, $Mg^{2+}$-ATPase, and $Ca^{2+}$-ATPase), together with total adenylate (TA) and adenylate energy charge (AEC), important indicators of environmental stress, in different brain regions, cerebrum and cerebellum, at gestation day (GD) 15 and 20.

We found that the concentration of serum thyroxine (T4), triiodothyronine (T3) and thyrotropin (TSH) in control maternal rats and their fetuses was significantly increased from GD 15 to 20. Earlier study from my laboratory has shown that a gradual increase of serum T4, T3 and TSH levels was recorded at GD 16 and 19 in control maternal rats and their fetuses (Ahmed, 2011). Gärtner, in 2009, reported that the demand of thyroid hormones (THs) increases during pregnancy to about 30–50% and the thyroid has to cope with this increase. These results can be due to the higher transfer of THs from pregnant females to their fetuses during pregnancy and/or more efficiency of thyroid gland to produce THs after birth (Jiskra et al., 2007; Ahmed et al., 2008; El-bakry et al., 2010; Ahmed, 2012a). The transplacental transport may also require deiodinases, transporters, sulfotransferases, and genomic and non-genomic actions (Loubière et al., 2010; Ahmed, 2011, 2012b). Several investigators (Ahmed et al., 2010, 2012; El-bakry et al., 2010; Ahmed, 2011, 2012a) stated that the gradual increase of TSH is necessary for the development of thyroid gland during this sensitive period. Thus, in the light of the above considerations, our data confirm that the adequate functioning of the maternal thyroid gland plays an important role in normal fetus development. In addition, the levels of serum growth hormone (GH) of control rat fetuses were markedly elevated in an age-dependent manner from GD 15 to 20. Consistent with our data, several authors considered the GH as a key factor controlling development (Wong et al., 2006; Ahmed et al., 2010, 2012). In fact, we have shown that the THs may regulate the growth and development, in part, affecting GH function and insulin-like growth factor (IGF) (Wasniewska et al., 2003; Ahmed et al., 2008, 2010, 2012; Ahmed, 2012a). Our data confirm an essential role of THs in the regulation of growth and development.

The administration of DOX (25 mg/kg) during the whole gestation period to female rats decreased serum T4 and T3 levels and increased levels of serum TSH at GD 15 and 20 of fetuses

**Table 4**
Effect of maternal DOX in total adenylate (TA) and adenylate energy charge (AEC) of their fetuses during the gestational period.

| Brain region | Periods | DXR (ml/25 g) | Total adenylates (TA) | | Adenylate energy charge (AEC) | |
|---|---|---|---|---|---|---|
| Cerebrum | GD 15 | 0 | 137.61 | | 0.67 | |
| | | 0.2 | 109.00 | −20.80% | 0.69 | +2.99% |
| | GD 20 | 0 | 182.39 | | 0.64 | |
| | | 0.2 | 76.32 | −58.16% | 0.71 | +10.93% |
| Cerebellum | GD 15 | 0 | 182.69 | | 0.70 | |
| | | 0.2 | 129.25 | −29.26% | 0.73 | +4.29% |
| | GD 20 | 0 | 219.88 | | 0.69 | |
| | | 0.2 | 97.80 | −55.52% | 0.77 | +11.60% |

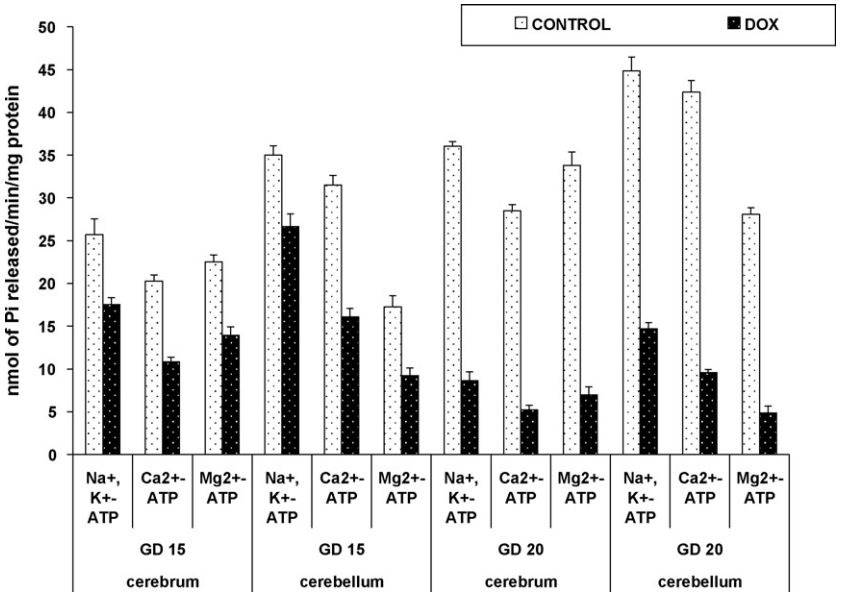


**Fig. 2.** Effect of maternal DOX on activities of $Na^+$, $K^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase in cerebrum and cerebellum of rat fetus at gestation days 15 and 20. Bars represent mean ± SE of six animals/group. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test are illustrated in Table 3. The change between DOX and control/parameter/period/brain region is highly significant ($P < 0.01$).

and dams. This administration was shown to affect T4/T3 ratio during the tested days and reflect the suppression of T4 to T3 conversion to compensate for the low levels of both hormones. The levels of fetal serum GH, at GD 15 and 20, were decreased in the maternal DOX group. We recently reported that thyroid functions during the development are dynamic and many external factors (*i.e.* dioxin) potentially influence the maternal–fetal–hypothalamic–pituitary–thyroid axis (HPTA) (Ahmed, 2011; Ahmed, 2012b). This result is reinforced by Madanat et al. (2007) who reported the chemotherapy-induced thyroid hypofunction, Rose et al. (2004) who recorded the chemotherapy-induced HPTA dysfunction (hypothyroidism, GH-deficiency or pubertal abnormality), van Leeuwen et al. (2000) who postulated that DOX may result in a relative GH-resistance, and McLeod et al. (2012) who undertook that higher serum TSH concentrations are generally associated with an increased risk of thyroid cancer. In line with these results, the late-effects of cancer therapy can cause growth retardation (Darzy and Shalet, 2003; Van den Bos et al., 2004) by inhibiting GH or IGF-I (Meacham, 2003). It is observed from the above mentioned results that a transient and moderate deficiency of maternal THs can have deleterious consequences on thyroid function of both mothers and their fetuses. Also, maternal THs deficiency may disturb the secretion of other pituitary hormones in their fetuses. These results indicated that the thyroid function is related to maternal doses. However, it remains to be seen whether higher concentrations of DOX might cause more persistent perturbations in THs during the development.

On the other hand, our data show that the levels of fetal nucleotides (ATP, ADP and AMP), the activities of fetal ATPases ($Na^+$, $K^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase), and the values of fetal TA in control group are markedly elevated in all investigated brain regions, cerebrum and cerebellum, in an age-dependent manner from GD 15 to 20. Several investigators have documented that adenosine plays an important regulatory role in the functioning, differentiation and survival of developing neural cells (Heilbronn et al., 1995; Bruno et al., 2002; Merighi et al., 2003; Ahmed et al., 2008). They hypothesized that this effect is dependent on extracellular adenosine concentrations, cell surface expression of different adenosine receptor subtypes, and signal transduction mechanisms activated following the binding of specific agonists. In developing rat brain, there is an increase in the activities of $Na^+$, $K^+$-ATPase (Valcana and Timiras, 1969; Bertoni and Siegel, 1978; Ahmed et al., 2010; Ahmed, 2012a), $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase (Ahmed et al., 2010; Ahmed, 2012a). The increases in pump activities are accompanied by a change in the brain's ionic composition (Valcana and Timiras, 1969; dos Reis-Lunardelli et al., 2007) and a greater number of functional sodium pump sites (Bertoni and Siegel, 1978). These results are in agreement with those of Lopez et al. (2002). The gradual increases of nucleotides and ATP-ases from GD 15 to 20 are very relevant for the development of cerebrum and cerebellum. In addition, these pump activities contribute to the maintenance of physiological levels of extracellular ATP/ADP/AMP and adenosine.

On the other hand, the activities of fetal ATP-ases may depend on the concentration of THs during the development. THs modulate the cellular sodium current, inward rectifying potassium current, sodium pump ($Na^+$, $K^+$-ATPase) and calcium pump ($Ca^{2+}$-ATPase) (Davis et al., 2010; Ahmed et al., 2010; Ahmed, 2012a). These data are supported by Zamoner et al. (2006) who emphasized that THs have membrane-initiated actions modulating $Ca^{2+}$ channels suggesting the presence of multiple sites of hormonal regulation and supporting a role for THs as modulators of signal transduction pathways in the CNS of rats. Similar data were reported by D'Arezzo et al. (2004), Volpato et al. (2004), Zamoner et al. (2005, 2007, 2008, 2011), Incerpi et al. (2005), Scapin et al. (2009, 2010) and Menegaz et al. (2010) who demonstrated that most of the nongenomic effects of THs involve $Ca^{2+}$-mediated cellular responses in different cell types. In agreement with our results, Bruno et al. (2003, 2005) elucidated that THs are associated with an increase in the adenosine transport in brain and that adenosine plays an important modulatory role in physiological situations. Also, the ATP-ases can regulate the ionic composition of the intra- and extracellular medium (Incerpi et al., 2005; Scapin et al., 2009, 2010). Hence, these observations lead us to conclude the following: (1) in normal state, the enzymatic (ATP-ases) activities can regulate the concentration of adenine nucleotides and (2) the nucleotides and adenosinergic system, under control of THs, play a crucial role for the differentiation of CNS.

Furthermore, in the current investigation, a decrease in the levels of fetal nucleotides (ATP, ADP and AMP), in the activities of fetal ATPase system ($Na^+$, $K^+$-ATPase, $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase)

and in the values of fetal TA was observed in cerebrum and cerebellum of maternal DOX group at GD 15 and 20 with respect to control ones. In particular, the $Na^+$, $K^+$-ATPase of cerebrum appears to be more sensitive to DOX treatment with respect to cerebellum at a later stage, and this higher sensitivity could be in line with its pivotal role in maintaining the ionic composition across the plasma membrane of excitable cells. Substantial agreement with our data is shown by several studies. Administration of DOX decreases the activity of ADP (Vidal et al., 1996), changes the ATP synthesis, changes the profile of energy substrate utilization and disturbs the energy transfer between sites of energy production and consumption (Shneyvays et al., 2001, 2002; Tokarska-Schlattner et al., 2006; Emanuelov et al., 2010). DOX has been shown to inhibit sarcolemmal $Na^+/Ca^{2+}$ exchange (Caroni et al., 1981) and $Na^+/K^+$ pump (Shneyvays et al., 2001). In both human and animal models, DOX treatment decreased the ATP/ADP ratio (Tokarska-Schlattner et al., 2005; Kumar et al., 2011; Octavia et al., 2012), inhibited sarcoplasmic reticulum $Ca^{2+}$-ATPase (Kumar et al., 2011) and impaired $Ca^{2+}$ homeostasis (Vidal et al., 1996) resulting in a depletion of high-energy phosphates (De Beer et al., 2001), general energy impairment (Kumar et al., 2011) with cytotoxic activity were also reported (Vidal et al., 1996).This state may reflect the depletion of high-energy phosphates (De Beer et al., 2001) and general energy impairment (Kumar et al., 2011). Interestingly, the DOX can exert these actions through four major mechanisms (Robert, 2007; Wallace, 2007; Simunek et al., 2009; Lal et al., 2010; Thorn et al., 2011): (1) binding to cellular membranes to alter fluidity and ion transport; (2) high-affinity binding to DNA through intercalation, with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission (Bertolatus et al., 1991) to induce mutations, chromosomal aberrations (Quiles et al., 2002) and maldevelopment (Hozayen, 2012); (3) inhibition of topoisomerase II; and (4) generation of semiquinone free radicals and oxygen free radicals such as superoxide anion through an enzyme-mediated reductive process (Bertolatus et al., 1991; Sterba et al., 2012). Many of doxorubicin's dose-limiting toxicities occur due to generation of ROS, resulting in oxidative stress (Hozayen, 2012) in whole body (Ahmed et al., 2005; Abdella and Ahmed, 2009). From the quoted papers and from our data we can hypothesize that the toxicity of DOX is due to depletion of nucleotides and ATP-ases as a first event, followed by impairment of the neural energetics and energy production.

On the other hand, in light of the current work, the fetal AEC increased in maternal treated group with respect to control ones. The AEC values are nearly similar, this supports the hypothesis that living organisms could obtain their required energy via different sources in order to survive. This aim can be achieved via degradation of body stores (protein and fat) to support energetic demands (El-Wardany et al., 2011), and in agreement with the fact that the cells need to keep the energy charge as high as possible as a mechanism for survival (Atkinson, 1970). In this context, animals (rats) can rely on decreasing basal metabolic rate via modulating thyroid activity and initiating the stored energetic power of the body (Ahmed, 2012a).

Our data showed that administration of DOX to maternal rat during gestation period severely affects the thyroid–brain axis leading to hypothyroid state, this, in turn, gives rise to a deficiency of nucleotides and adenosinergic enzymes in fetal cerebrum and cerebellum. The rate of ATP synthesis decreases significantly in the hypothyroid state (Wrutniak-Cabello et al., 2001; Dave et al., 2006). This is confirmed by our previous studies showing that hypothyroidism during development induces a decrease in the activity of adenosine-metabolizing enzymes ($Na^+$, $K^+$-ATPase, $Mg^{2+}$-ATPase and $Ca^{2+}$-ATPase) in different brain regions (Ahmed et al., 2010; Ahmed, 2012a). This in turn could result in impaired nerve transmission (Gerges and Alkadhi, 2004; Billimoria et al., 2006; Katyare et al., 2006; Ahmed et al., 2008) and generally CNS dysfunction

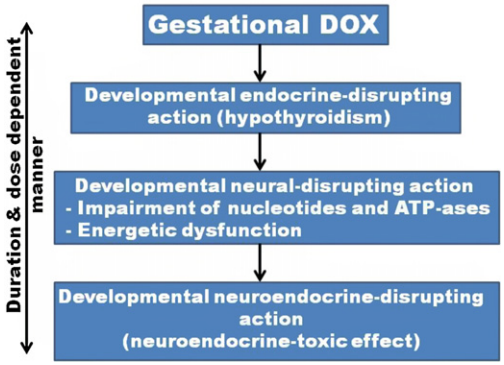


**Fig. 3.** Schematic diagram for the toxicology of DOX on the developmental neuroendocrine system.

(Mussa et al., 2001; Dave et al., 2006). The $Ca^{2+}$-ATPase activity is decreased in the hypothyroid rat brain (Dave et al., 2006). These effects are in agreement with the clinical manifestations ascribed to hypothyroidism in rats (Bruno et al., 2005) and are concomitant with our previous publication (Ahmed et al., 2008). In the light of these observations and the previous results, we can conclude that the CNS is particularly sensitive to any disturbances during pregnancy in energy generation and even a short-term interruption may lead to long-lasting and irreversible damage (fetal brain energetic dysfunction) as reported in recent investigations.

## 5. Conclusion and recommendations

The transplacental transfer of DOX was demonstrated in a fetus rat model caused impairment in the development of cerebrum and cerebellum via THs dysfunction and this may delay the growth and differentiation of the neuroendocrine system. Thus, DOX may act as endocrine- and neural-disrupting actions on the development of THs–brain axis (Fig. 3). These drastic effects depend on the type, amount, threshold dose, and animal species. The neuroendocrine-disruption associated with DOX treatment may limit the therapeutic efficiency of this drug against cancer because any minor changes in thyroid function during the development can result in brain damage (Zoeller and Crofton, 2005; Kagami et al., 2010; Ahmed, 2012a,b; Ahmed and Ahmed, 2012). By the way, the clinical studies showed that maternal TH deficiency during the first trimester of pregnancy can affect the outcome of human neurodevelopment (Pop et al., 2003). More importantly, Matalon et al. (2004) undertook that DOX facilitates or induces apoptosis and thus may damage embryonic development and placental function. For these reasons, we suggest that thyroid function should be monitored carefully during and shortly after treatment with chemotherapy, since low THs concentrations in mothers can have severe consequences for the fetal growth and development, particularly CNS. We advise always at least annual evaluation for T4, T3 and TSH for DOX-mothers and their children, and following the GH, growth rate, pubertal abnormalities, and other hypothalamic dysfunction for these children because to date, clinicians are reluctant to prescribe cytotoxic drugs in pregnant women since long term safety data are lacking. Clearly, research in this field is still in its infancy, with several important and challenging issues remaining to be addressed.

## Conflict of interest

The authors report no conflict of interest.

## Acknowledgments

We gratefully acknowledge a Lab Visit Grant from the European Society for Endocrinology – UK to R.G. Ahmed and Beni-Suef University who provided invaluable help to this work. The financial support from the Italian Ministry for Education, University and Research, General Management for International Research is also acknowledged.

## References

Abdella, E.M., Ahmed, R.R., 2009. Suppression of doxorubicin apoptotic, histopathologic, mutagenic and oxidative stress effects in mice bone marrow and testis tissues by aqueous rosemary leaves extract. Acta Biologica Paranaense Curitiba 38 (1–2), 35–57.

Ahmed, H.H., Mannaa, F., Elmegeed, G.A., Doss, S.H., 2005. Cardioprotective activity of melatonin and its novel synthesized derivatives on doxorubicin-induced cardiotoxicity. Bioorganic & Medicinal Chemistry 13 (5), 1847–1857.

Ahmed, O.M., Abd El-Tawab, S.M., Ahmed, R.G., 2010. Effects of experimentally induced maternal hypothyroidism and hyperthyroidism on the development of rat offspring. I. The development of the thyroid hormones-neurotransmitters and adenosinergic system interactions. International Journal of Developmental Neuroscience 28, 437–454.

Ahmed, O.M., Ahmed, R.G., 2012. A new look at hypothyroidism. In: InTech Open Access Publisher, University Campus, STeP Ri Slavka Krautzeka 83/A, 51000 Rijeka, Croatia, pp. 1–20.

Ahmed, O.M., Ahmed, R.G., El-Gareib, A.W., El-Bakry, A.M., Abd El-Tawaba, S.M., 2012. Effects of experimentally induced maternal hypothyroidism and hyperthyroidism on the development of rat offspring. II. The developmental pattern of neurons in relation to oxidative stress and antioxidant defense system. International Journal of Developmental Neuroscience 30, 517–537.

Ahmed, O.M., El-Gareib, A.W., El-bakry, A.M., Abd El-Tawab, S.M., Ahmed, R.G., 2008. Thyroid hormones states and brain development interactions. International Journal of Developmental Neuroscience 26 (2), 147–209.

Ahmed, R.G., 2011. Perinatal 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure alters developmental neuroendocrine system. Food and Chemical Toxicology 49, 1276–1284.

Ahmed, R.G., 2012a. Maternal-newborn thyroid dysfunction. In: LAP LAMBERT Academic Publishing GmbH & Co. KG, Heinrich-Böcking-Str. 6-8, 66121 Saarbrücken, Germany, pp. 1–369. ISBN: 978-3-8484-8688-5.

Ahmed, R.G., 2012b. Maternal–fetal thyroid interactions. In: InTech Open Access Publisher, University Campus, STeP Ri Slavka Krautzeka 83/A, 51000 Rijeka, Croatia, pp. 125–156.

Antunes, L., Takhashi, C., 1999. Modulatory effects of curcumin on the chromosomal damage induced by doxorubicin in Chinese hamster ovary cells. Teratogenesis, Carcinogenesis, and Mutagenesis 19, 1–8.

Arai, M., Yoguchi, A., Takizawa, T., Yokoyama, T., Kanda, T., Kurabayashi, M., Nagai, R., 2000. Mechanism of doxorubicin-induced inhibition of sarcoplasmic reticulum $Ca^{2+}$-ATPase gene transcription. Circulation Research 86, 8–14.

Atkinson, D.E., 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry 7, 4030–4034.

Atkinson, D.E., 1970. Enzymes as control elements in metabolic regulation. In: Boyer, P.D. (Ed.), The Enzymes. Academic Press, New York, pp. 461–489.

Barber, H.R., 1981. Fetal and neonatal effects of cytotoxic agents. Obstetrics & Gynecology 58 (Suppl.), 41–47.

Bertolatus, J.A., Klinzman, D., Bronsema, D.A., Ridnour, L., Oberley, L.W., 1991. Evaluation of the role of reactive oxygen species in doxorubicin hydrochloride nephrosis. Journal of Laboratory and Clinical Medicine 118 (5), 435–445.

Bertoni, J.M., Siegel, G.J., 1978. Development of $Na^+$, $K^+$-ATPase in rat cerebrum: correlation with $Na^+$-dependent phosphorylation and $K^+$-paranitrophenylphosphatase. Journal of Neurochemistry 31 (6), 1501–1511.

Bigotte, L., Olsson, Y., 1984. Cytotoxic effects of adriamycin on the central nervous system of the mouse-cytofluorescence and electron-microscopic observations after various modes of administration. Acta Neurologica Scandinavica, Supplementum 100, 55–67.

Billimoria, F.R., Dave, B.N., Katyare, S.S., 2006. Neonatal hypothyroidism alters the kinetic properties of $Na^+$, $K^+$-ATPase in synaptic plasma membranes from rat brain. Brain Research Bulletin 70, 55–61.

Bruno, A.N., Bonan, C.D., Wofchuk, S.T., Sarkis, J.J.F., Battastini, A.M.O., 2002. ATP diphosphohydrolase (NTPDase I) in rat hippocampal slices and effect of glutamate on enzyme activity in different phases of development. Life Sciences 71, 215–225.

Bruno, A.N., Da Silva, R.S., Bonan, C.D., Battastini, A.M., Barreto-chaves, M.L., Sarkis, J.J., 2003. Hyperthyroidism modifies ecto-nucleotidase activities in synaptosomes from hippocampus and cerebral cortex of rats in different phases of development. International Journal of Developmental Neuroscience 21 (7), 401–408.

Bruno, A.N., Ricachenevsky, F.K., Pochmann, D., Bonan, C.D., Battastini, A.M., Barreto-Chaves, M.L., Sarkis, J.J., 2005. Hypothyroidism changes adenine nucleotide hydrolysis in synaptosomes from hippocampus and cerebral cortex of rats in different phases of development. International Journal of Developmental Neuroscience 23 (1), 37–44.

Canadian Council on Animal Care (CCAC), 1993. Guide to the Care and Use of Experimental Animals, vol. 2. CCAC, Ottawa, Ontario, Canada.

Carageorgiou, H., Pantos, C., Zarros, A., Stolakis, V., Mourouzis, I., Cokkinos, D., Tsakiris, S., 2007. Changes in acetylcholinesterase, $Na^+$, $K^+$-ATPase, and $Mg^{2+}$-ATPase activities in the frontal cortex and the hippocampus of hyper- and hypothyroid adult rats. Metabolism: Clinical and Experimental 56 (8), 1104–1110.

Cardonick, E., Iacobucci, A., 2004. Use of chemotherapy during human pregnancy. The Lancet Oncology 5, 283–291.

Caroni, P., Villani, F., Carafoli, E., 1981. The cardiotoxic antibiotic doxorubicin inhibits the $Na^+/Ca^{2+}$ exchange of dog heart sarcolemmal vesicles. FEBS Letters 130, 184–186.

Chan, K., Delfert, D., Junguer, K.D., 1986. A direct colorimetric assay for $Ca^{+2}$-ATPase activity. Analytical Biochemistry 157, 375–380.

D'Arezzo, S., Incerpi, S., Davis, F.B., Acconcia, F., Marino, M., Farías, R.N., Davis, P.J., 2004. Rapid nongenomic effects of 3,5,3′-triiodo-L-thyronine on the intracellular pH of L-6 myoblasts are mediated by intracellular calcium mobilization and kinase pathways. Endocrinology 145, 5694–5703.

Darzy, K.H., Shalet, S.M., 2003. Radiation induced growth hormone deficiency. Hormone Research 59, 1–11.

Dave, B.N., Katyare, S.S., Billimoria, F.R., 2006. Effect of neonatal hypothyroidism on $Ca^{2+}$-ATPase activity in the rat brain. Neuroscience Letters 395, 33–36.

Davis, P.J., Zhou, M., Davis, F.B., Lansing, L., Mousa, S.A., Lin, H.-Y., 2010. Mini-review: cell surface receptor for thyroid hormone and nongenomic regulation of ion fluxes in excitable cells. Physiology & Behavior 99, 237–239.

De Beer, E.L., Bottone, A.E., Voest, E.E., 2001. Doxorubicin and mechanical performance of cardiac trabeculae after acute and chronic treatment: a review. European Journal of Pharmacology 415, 1–11.

De Luca-Abbott, S., Creese, R.G., Lewis, G.D., Wells, R.M.G., 2000. Adenylate energy charge and total adenylate nucleotide pool as biomarkers of sublethal stress in the cockle, *Austrovenus stutchburyi*. Australasian Journal of Ecotoxicology 6, 35–44.

dos Reis-Lunardelli, E.A., Castro, C.C., Bavaresco, C., Coitinho, A.S., da Trindade, L.S., Perrenoud, M.F., Roesler, R., Sarkis, J.J., de Souza Wyse, A.T., Izquierdo, I., 2007. Effects of thyroid hormones on memory and on $Na^+$, $K^+$-ATPase activity in rat brain. Current Neurovascular Research 4 (3), 184–193.

El-bakry, A.M., El-Ghareeb, A-W.A., Ahmed, R.G., 2010. Comparative study of the effects of experimentally-induced hypothyroidism and hyperthyroidism in some brain regions in albino rats. International Journal of Developmental Neuroscience 28, 371–389.

Elekwa, I., Monanu, M.O., Anosike, E.O., 2005. In vitro effects of aqueous extracts of zanthoxylum macrophylla roots on adenosine triphosphatases in erythrocytes of different genotypes. Biokemistri 17 (1), 19–25.

El-Wardany, I., El-Daly, E.F., Abd El-Gawad, A.H., Hemid, A.E.A., Abd El-Azeem, N.A., 2011. Effect of thyroid activity modulation on some histological and biochemical aspects in broiler chicks. American-Eurasian Journal of Agricultural & Environmental Sciences 11 (6), 893–902.

Emanuelov, A.K., Shainberg, A., Chepurko, Y., Kaplan, D., Sagie, A., Porat, E., Arad, M., Hochhauser, E., 2010. Adenosine $A_3$ receptor-mediated cardioprotection against doxorubicin-induced mitochondrial damage. Biochemical Pharmacology 79, 180–187.

Gärtner, R., 2009. Thyroid disorders during pregnancy. The Deutsche Medizinische Wochenschrift 134 (3), 83–86.

Gerges, N.Z., Alkadhi, K.A., 2004. Hypothyroidism impairs late LTP in CA1 region but not in dentate gyrus of the intact rat hippocampus: MAPK involvement. Hippocampus 14, 40–45.

Gilbert, M.E., Sui, L., 2006. Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. Brain Research 1069 (1), 10–22.

Heilbronn, A., Maienschein, V., Carstensen, K., Gann, W., Zimmermann, H., 1995. Crucial role of ecto-5′-nucleotidase in differentiation and survival of developing neural cells. Neuroreport 7 (1), 257–261.

Hesketh, J.E., London, J.B., Reading, H.W., Glen, A.I., 1978. The effect of lithium treatment on erythrocyte membrane ATPase activities and erythrocyte ion content. British Journal of Clinical Pharmacology 5, 323–329.

Horn, S., Heuer, H., 2010. Thyroid hormone action during brain development: more questions than answers. Molecular and Cellular Endocrinology 315 (1–2), 19–26.

Hozayen, W.G., 2012. Effect of hesperidin and rutin on doxorubicin induced testicular toxicity in male rats. International Journal of Food Sciences and Nutrition 1 (1), 31–42.

Hozayen, W.G., Abou Seif, H.S., 2011. Protective effects of rutin and hesperidin against doxorubicin-induced lipodystrophy and cardiotoxicity in albino rats. The Journal of American Science 7 (12), 765–775, http://www.nacb.org/lmpg/thyroid_LMPG_Word.stm (accessed January 2005).

Incerpi, S., Scapin, S., D'Arezzo, S., Spagnuolo, S., Leoni, S., 2005. Short-term effect of thyroid hormone in prenatal development and cell differentiation. Steroids 70, 434–443.

Jiskra, J., Límanová, Z., Potluková, E., Antosová, M., 2007. The importance of screening for thyroid dysfunction during pregnancy: pathophysiological background and practical implications. Casopis Lekaru Ceskych 146 (11), 827–833.

Kagami, K., Nishigori, H., Nishigori, H., 2010. Effects of prenatal exposure to antithyroid drugs on imprinting behavior in chicks. Physiology & Behavior 101, 297–301.

Katyare, S.S., Billimoria, F.R., Dave, B.N., 2006. Effect of neonatal hypothyroidism on the kinetic properties of $Na^+$, $K^+$-ATPase from rat brain microsomes. Neuroendocrinology 18, 361–366.
Kawasaki, N., Lee, J.D., Shimizu, H., Ueda, T., 1996. Long term L-carnitine treatment prolongs the survival in rats with adriamycin induced heart failure. Journal of Cardiac Failure 2, 293–299.
Koibuchi, N., 2009. Animal models to study thyroid hormone action in cerebellum. Cerebellum 8, 89–97.
Koibuchi, N., Chin, W.W., 2000. Thyroid hormone action and brain development. Trends in Endocrinology & Metabolism 11, 123–128.
Kumar, S.N., Konorev, E.A., Aggarwal, D., Kalyanaraman, B., 2011. Analysis of proteome changes in doxorubicin-treated adult rat cardiomyocyte. Journal of Proteomics 74, 683–697.
Lal, S., Mahajan, A., Chen, W.N., Chowbay, B., 2010. Pharmacogenetics of target genes across doxorubicin disposition pathway: a review. Current Drug Metabolism 11, 115–128.
Lopez, L.B., Quintas, L.E.M., Noël, F., 2002. Influence of development on $Na^+$-$K^+$-ATPase expression: isoform- and tissue-dependency. Comparative Biochemistry and Physiology. Part A 131, 323–333.
Loubière, L.S., Vasilopoulou, E., Bulmer, J.N., Taylor, P.M., Stieger, B., Verrey, F., McCabe, C.J., Franklyn, J.A., Kilby, M.D., Chan, S.-Y., 2010. Expression of thyroid hormone transporters in the human placenta and changes associated with intrauterine growth restriction. Placenta 31, 295–304.
Madanat, L-M.S., Lähteenmäkia, P.M., Alinb, J., Salmia, T.T., 2007. The natural history of thyroid function abnormalities after treatment for childhood cancer. European Journal of Cancer 43, 1161–1170.
Maes, M., Mommen, K., Hendrickx, D., Peeters, D., D'Hondt, P., Ranjan, R., De Meyer, F., Scharpé, S., 1997. Components of biological variation, including seasonality, in blood concentrations of TSH, FT3, FT4, PRL, cortisol and testosterone in healthy volunteers. Clinical Endocrinology (Oxford) 46 (5), 587–598.
Mandel, S.J., Brent, G.A., Larsen, P.R., 1993. Levothyroxine therapy in patients with thyroid disease. Annals of Internal Medicine 119, 492–502.
Matalon, S.T., Ornoy, A., Lishner, M., 2004. Review of the potential effects of three commonly used antineoplastic and immunosuppressive drugs (cyclophosphamide, azathioprine, doxorubicin on the embryo and placenta). Reprod. Toxicol. 18, 219–230.
McLeod, D.S., Watters, K.F., Carpenter, A.D., Ladenson, P.W., Cooper, D.S., Ding, E.L., 2012. Thyrotropin and thyroid cancer diagnosis: a systematic review and dose-response meta-analysis. The Journal of Clinical Endocrinology & Metabolism 97 (8), 2682–2692.
Meacham, L., 2003. Endocrine late effects of childhood cancer therapy. Current Problems in Pediatric and Adolescent Health Care 33 (7), 217–242.
Menegaz, D., Barrientos-Duran, A., Kline, A., Silva, F.R., Norman, A.W., Mizwicki, M.T., Zanello, L.P., 2010. 1alpha, 25(OH)2-vitamin D3 stimulation of secretion via chloride channel activation in Sertoli cells. The Journal of Steroid Biochemistry and Molecular Biology 119, 127–134.
Menegola, E., Broccia, M.L., Di Renzo, F., 2001. Teratogenic effects of doxorubicin in rats at midgestation and at term. Teratogenesis, Carcinogenesis, and Mutagenesis 21, 283–293.
Merighi, S., Mirandola, P., Varani, K., Gessi, S., Leung, E., Baraldi, P.G., Tabrizi, M.A., Borea, P.A., 2003. A glance at adenosine receptors: novel target for antitumor therapy. Pharmacology & Therapeutics 100, 31–48.
Mussa, G.C., Mussa, F., Bretto, R., Zambilli, M.C., Silvestro, L., 2001. Influence of thyroid in nervous system growth. Minerva Pediatrica 53, 325–353.
Octavia, Y., Tocchetti, C.G., Gabrielson, K.L., Janssens, S., Crijns, H.J., Moens, A.L., 2012. Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. Journal of Molecular and Cellular Cardiology 52, 1213–1225.
PC-STAT, 1985. One way analysis of variance. Version 1A(c) copyright. Programs coded by Roa, M., Blane, K., Zonneberg, M. Univ. of Georgia, USA.
Pop, V.J., Brouwers, E.P., Vader, H.L., Vulsma, T., van Baar, A.L., de Vijlder, J.J., 2003. Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. Clinical Endocrinology 59, 282–288.
Prahalathan, C., Selvakumar, E., Varalakshmi, P., Kumarasamy, P., Saravanan, R., 2006. Salubrious effects of lipoic acid against adriamycin-induced clastogenesis and apoptosis in wistar rat bone marrow cells. Toxicology 15 (3), 225–232.
Quiles, J.L., Huertas, J.R., Battino, M., Mataix, J., Ramirez, T.M.C., 2002. Antioxidant nutrients and adriamycin toxicity. Toxicology 180 (1), 79–95.
Reutens, A.T., 1995. Evaluation and application of a highly sensitive assay for serum growth hormone (GH) in the study of adult GH deficiency. The Journal of Clinical Endocrinology & Metabolism 80 (2), 480–485.
Rico, E.P., Senger, M.R., Fauth, M.G., Dias, R.D., Bogo, M.R., Bonan, C.D., 2003. ATP and ADP hydrolysis in brain membranes of zebrafish (*Danio rerio*). Life Sciences 73, 2071–2082.
Robert, J., 2007. Preclinical assessment of anthracycline cardiotoxicity in laboratory animals: predictiveness and pitfalls. Cell Biology and Toxicology 23, 27–37.
Rose, S.R., Schreiber, R.E., Kearney, N.S., Lustig, R.H., Danish, R.K., Burghen, G.A., Hudson, M.M., 2004. Hypothalamic dysfunction after chemotherapy. Journal of Pediatric Endocrinology and Metabolism 17, 55–66.
Scapin, S., Leoni, S., Spagnuolo, S., Fiore, A.M., Incerpi, S., 2009. Short-term effects of thyroid hormones on the $Na^+$-$K^+$ATPase activity of chick embryo hepatocytes during development: focus on signal transduction. American Journal of Physiology: Cell Physiology 296, C4–C12.
Scapin, S., Leoni, S., Spagnuolo, S., Gnocchi, D., De Vito, P., Luly, P., Pedersen, J.Z., Incerpi, S., 2010. Short-term effects of thyroid hormones during development: focus on signal transduction. Steroids 75, 576–584.
Shneyvays, V., Mamedova, L., Korkus, A., Shainberg, A., 2002. Cardiomyocyte resistance to doxorubicin mediated A3 adenosine receptor. Journal of Molecular and Cellular Cardiology 34, 493–507.
Shneyvays, V., Mamedova, L., Zinman, T., Jacobson, K., Shainberg, A., 2001. Activation of $A_3$ adenosine receptor protects against doxorubicin-induced cardiotoxicity. Journal of Molecular and Cellular Cardiology 33, 1249–1261.
Simunek, T., Sterba, M., Popelova, O., Adamcova, M., Hrdina, R., Gersl, V., 2009. Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron. Pharmacol Reports 61, 154–171.
Sterba, M., Popelova, O., Vavrova, A., Jirkovsky, E., Kovarikova, P., Gersl, V., Simunek, T., 2012. Oxidative stress, redox signaling and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. Antioxidants & Redox Signaling (July) (Epub ahead of print).
Thakur, C., Saikia, T.C., Yadav, R.N., 1997. Total serum levels of triiodothyronine (T3), thyroxine (T4) and thyrotropin (TSH) in school going children of Dibrugarh district: an endemic goiter region of Assam. Indian Journal of Physiology and Pharmacology 41 (2), 167–170.
Thorn, C.F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T.E., Altman, R.B., 2011. Doxorubicin pathways: pharmacodynamics and adverse effects. Pharmacogenetics and Genomics 21 (7), 440–446.
Tokarska-Schlattner, M., Zaugg, M., Zuppinger, C., Wallimann, T., Schlattner, U., 2006. New insights into doxorubicin-induced cardiotoxicity: the critical role of cellular energetics. Journal of Molecular and Cellular Cardiology 41, 389–405.
Tokarska-Schlattner, M., Zaugg, M., da Silva, R., Lucchinetti, E., Schaub, M.C., Wallimann, T., Schlattner, U., 2005. Acute toxicity of doxorubicin on isolated perfused heart: response of kinases regulating energy supply. American Journal of Physiology: Heart and Circulatory Physiology 289, H37–H47.
Valcana, T., Timiras, P.S., 1969. Effect of hypothyroidism on ionic metabolism and $Na^+$-$K^+$ activated ATP phosphohydrolase activity in the developing rat brain. Neurochemistry 16 (3), 935–943.
Van Calsteren, K., Hartmann, D., Van Aerschot, L., Verbesselt, R., Van Bree, R., D'Hooge, R., Amant, F., 2009. Vinblastine and doxorubicin administration to pregnant mice affects brain development and behaviour in the offspring. Neurotoxicology 30, 647–657.
Van den Bos, C., Heinen, R.C., Sukel, M., van der Pal, H.J.H., Geenen, M.M., 2004. Screening for late effects in survivors of childhood cancer: growth hormone deficiency from a pediatric oncologist's point of view. Growth Hormone & IGF Research 14, 125–128.
van Leeuwen, B.L., Kamps, W.A., Jansen, H.W., Hoekstra, H.J., 2000. The effect of chemotherapy on the growing skeleton. Cancer Treatment Reviews 26, 363–376.
Vidal, R.F., Eksborg, S., Sundberg, M., Carlberg, M., Elfsson, B., Andersson, B.S., 1996. Doxorubicin- and daunorubicin-induced energy deprivation and nucleotide degradation in isolated cardiomyocytes. Toxicology 114, 1–10.
Volpato, K.C., Menegaz, D., Leite, L.D., Barreto, K.P., de Vilhena, G.E., Silva, F.R., 2004. Involvement of $K^+$ channels and calcium-dependent pathways in the action of T3 on amino acid accumulation and membrane potential in Sertoli cells of immature rat testis. Life Sciences 74, 1277–1288.
Wallace, K.B., 2007. Adriamycin-induced interference with cardiac mitochondrial calcium homeostasis. Cardiovascular Toxicology 7, 101–107.
Wasniewska, M., De Luca, F., Cassio, A., Oggiaro, N., Gianino, P., Delvecchio, M., Aiazzi, R., Stoppioni, V., Lombardo, F., Messina, M.F., Valenzise, M., Arrigo, T., 2003. In congenital hypothyroidism bone maturation at birth may be a predictive factor of psychomotor development during the first year of life irrespective of other variables related to treatment. European Journal of Endocrinology 149, 1–6.
Wong, A.O., Zhou, H., Jiang, Y., Ko, W.K., 2006. Feedback regulation of growth hormone synthesis and secretion in fish and the emerging concept of intrapituitary feedback loop. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 144 (3), 284–305.
Wrutniak-Cabello, C., Casas, F., Cabello, G., 2001. Thyroid hormone action in mitochondria. Journal of Molecular Endocrinology 26, 67–77.
Zamoner, A., Barreto, K.P., Filho, D.W., Sell, F., Woehl, V.M., Guma, F.C., Silva, F.R., Pessoa-Pureur, R., 2007. Hyperthyroidism in the developing rat testis is associated with oxidative stress and hyperphosphorylated vimentin accumulation. Molecular and Cellular Endocrinology 267, 116–126.
Zamoner, A., Corbelini, P.F., Funchal, C., Menegaz, D., Silva, F.R., Pessoa-Pureur, R., 2005. Involvement of calcium-dependent mechanisms on the action of T3 in the in vitro phosphorylation of vimentin of immature rat testis. Life Sciences 77, 3321–3335.
Zamoner, A., Funchal, C., Heimfarth, L., Silva, F.R., Pureur, R.P., 2006. Short-term effects of thyroid hormones on cytoskeletal proteins are mediated by GABAergic mechanisms in slices of cerebral cortex from young rats. Cellular and Molecular Neurobiology 26, 209–224.
Zamoner, A., Heimfarth, L., Oliveira, L.S., Royer, C., Silva, F.R., Pessoa-Pureur, R., 2008. Nongenomic actions of thyroxine modulate intermediate filament phosphorylation in cerebral cortex of rats. Neurosciences 156, 640–652.
Zamoner, A., Pessoa-Pureur, R., Silva, F.R., 2011. Membrane-initiated actions of thyroid hormones on the male reproductive system. Life Sciences 89, 507–514.
Zoeller, R.T., 2004. Editorial: local control of the timing of thyroid hormone action in the developing human brain. The Journal of Clinical Endocrinology & Metabolism 89 (7), 3114–3116.

Zoeller, R.T., 2006. Collision of basic and applied approaches to risk assessment of thyroid toxicants. Annals of the New York Academy of Sciences 1076 (1), 168–190.
Zoeller, R.T., 2007. Environmental chemicals impacting the thyroid: targets and consequences. Thyroid 17 (9), 811–817.
Zoeller, R.T., 2008. Environmental neuroendocrine and thyroid disruption: relevance for reproductive medicine? Fertility and Sterility 89 (2 (Suppl. 1)), e99–e100.
Zoeller, R.T., Crofton, K.M., 2005. Mode of action: developmental thyroid hormone insufficiency-neurological abnormalities resulting from exposure to propylthiouracil. Critical Reviews in Toxicology 35, 771–781.
Zoeller, R.T., Rovet, J., 2004. Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings. Neuroendocrinology 16, 809–818.